MORPHOLOGICAL FACTORS RELATING TO THE DEGRADATION OF WOOD FIBRES BY CELLULASE PREPARATIONS

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SUMMARY

In connection with the results obtained by studying the attack on Valonia ventricosa by helicase preparations (WARDROP & JUTTE 1968) further investigations were carried out to obtain a better understanding of the morphological factors relating to the degradation of wood fibres by cellulase preparations. To this end, the effect of degradation by cellulase preparations on Scots pine, beech and ash was studied, with both light and electron microscopy. Both unattacked and soft-rotted wood was used, either intact or as macerated fibres and tracheids. Moreover artificial lignification was used on the isolated wood elements and the Valonia ventricosa fragments before treatment with cellulases. The results obtained suggest that the form of soft rot cavities can be explained by the crystalline nature of the cellulose microfibrils.

1. INTRODUCTION

The influence of wood destroying fungi on the structure and properties of wood has been reviewed extensively in the literature, *e.g.* BAILEY & VESTAL (1937), FINDLAY & SAVORY (1954), SAVORY (1954), MEIER (1955), CARTWRIGHT & FINDLAY (1958), LEVI (1962), COURTOIS (1963, 1965), LIESE (1964, 1965), LEVY (1965), COWLING (1965 a, b).

Amongst the wood destroying fungi, the "soft rot" type gives the most spectacular destruction, because it makes characteristic cavities with a regular geometrical form in the cell walls, especially in the S2 layer (*fig. 1*). BAILEY & VESTAL (1937) in their description of this type of attack recognize that "there appear to be two determined planes of enzymatic activity in the secondary walls of tracheary cells and fibres: (1) oriented parallel to the long axis of the chain molecules and (2) oriented at an angle of from 20–25 degrees to this axis". By measurements of the angle of intersection of the two principal planes obtained from nine species and genera of seven different families, including one gymnosperm, they found an average angle of 22.3 degrees.

In a recent study of the degradation by cellulase of vesicles of *Valonia ventricosa*, it was noted by WARDROP & JUTTE (1968) that the enzyme caused the separation from the vesicle wall of microfibrils ca. 200 Å wide, which subsequently broke down into fibrillar subunits of ca. 40 Å wide. At the same time

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the microfibrils acquired oblique or pointed ends (*fig. 2*). These oblique planes made an angle of 23° with the microfibril axis. FRANKE & FALK (1968) in a study of the degradation of the *Valonia macrophysa* cell wall with pectinase preparations observed a similar breakdown to elementary fibrils, but did not observe the development of pointed ends in the microfibrils. Because of this pattern of breakdown it was postulated by WARDROP & JUTTE (1968) that similar factors determined the development of erosion patterns in wood caused by soft rot fungi and the development of the oblique terminal planes in the microfibrils of *Valonia*. In both instances the oblique planes of degradation were consistent with erosion along planes of the crystal lattice of cellulose as was suggested originally by BAILEY & VESTAL (1937) and by FREY-WYSSLING (1938). The former authors state that the illustrations of partially acetylated fibres published by HESS (1928), KANAMARU (1934) and others indicate that acetylation of cellulose tends to proceed along similar planes.

In view of the above observations it was considered of interest to study the effect of cellulase preparations on wood of angiosperms and of gymnosperms and on tracheids or fibres isolated by delignification. Attention was also directed to the influence of the level of lignification of the cell wall on the course of degradation by cellulase. This was done by comparing the attack by cellulase on tension wood with that on normal wood of angiosperms, since in tension wood the fibres possess a lower level of lignification than in normal wood and the gelatinous layer is mostly unlignified or only slightly so.

A further approach to this problem was made by studying the effect of artificial "lignification" on isolated cells. For this purpose the attack of cellulase on *Valonia* and isolated (delignified) fibres and tracheids was compared with the attack on similar cells after artificial lignification by methods such as those used by SIEGEL (1955) and by SOLBERG & HIGINBOTHAM (1957).

2. MATERIALS AND METHODS

2.1. Plant specimens

Pinus sylvestris (Scots Pine). Sound air dried wood and air dried wood from poles treated with preservative was used. The latter had been partly buried in the ground for about 20 years and in it heavy "soft rot" attack had taken place. The fungus causing the soft rot was not isolated and identified.

Fagus sylvatica (Beech). Normal wood and tension wood taken from the same growth region at a distance of about 15 cm from the pith was used.

Fraxinus excelsior (Ash). Only the late wood was used. The sampling of both normal wood and tension wood was carried out in the same way as with Beech.

Valonia ventricosa. Prepared as described by WARDROP & JUTTE (1968).

2.2. Enzyme preparations

The following enzyme preparations have been used:

Trichoderma viride cellulase ("Onozutia", Japan), a crude preparation. This was a yellowish powder and was used as a 1 per cent aqueous solution in acetate

buffer (pH 5.0). The solutions were centrifuged before use to remove diatoms contained in the preparation.

Helicase, as used in the investigation of WARDROP & JUTTE (1968). This preparation gave a clear solution and was used in 1 per cent concentration in distilled water.

Both preparations were used at 38–40° C. A drop of toluene was added to prevent microbial contamination.

2.3. Examination of specimens by light microscopy

For this purpose a Zeiss Photomicroscope fitted with polarizing interference and fluorescence attachments was used. Measurements of phase difference were carried out at a wavelength of 546 millimicrons using the polarizing system with a de Sénarmont compensator. The course of attack by the enzyme preparations on single fibres or tracheids was followed by mounting a suspension of the cells in the enzyme in a welled slide on a heating stage and observing the cells between crossed nicols. The cell under observation in each series studied was oriented at 45° to the plane of polarization. The wood was macerated by immersing of

Figs. 1. and 3–8. *Pinus sylvestris* L. (Scots Pine) showing attack by soft rot fungi in the late wood.

Fig. 1. Tangential section of tracheid with characteristic "soft rot" pattern. Polarized light between crossed nicols.

Fig. 2. Microfibril of *Valonia ventricosa* after 10 days of helicase treatment showing pointed end (24°). Negatively stained with phosphotungstic acid, electronmicrograph.

Fig. 3. Transverse section. In the degraded S2 layer hyphae can be detected; the level of fluorescence of the cell wall in the attacked cells appears lower than in adjacent uninfected ones. Fluorescence photomicrograph.

Fig. 4. Transverse section of tracheid, showing in the S2 layer a nearly circularhole with a light layer of modified lignin and matrix and with a large irregular hole containing light debris.
The S1 and S3 layers are not attacked. Electronmicrograph; araldite embedding.

Fig. 5. Longitudinal section through secondary wall of tracheid, showing a truly median section through the conical region of a cavity in the S2 layer. Electron micrograph, methacrylate embedded.

Fig. 6. Longitudinal section through secondary wall of tracheid showing a cavity that has not been cut through the centre and which is partly filled with modified cell wall substances. Electron micrograph, araldite embedded.

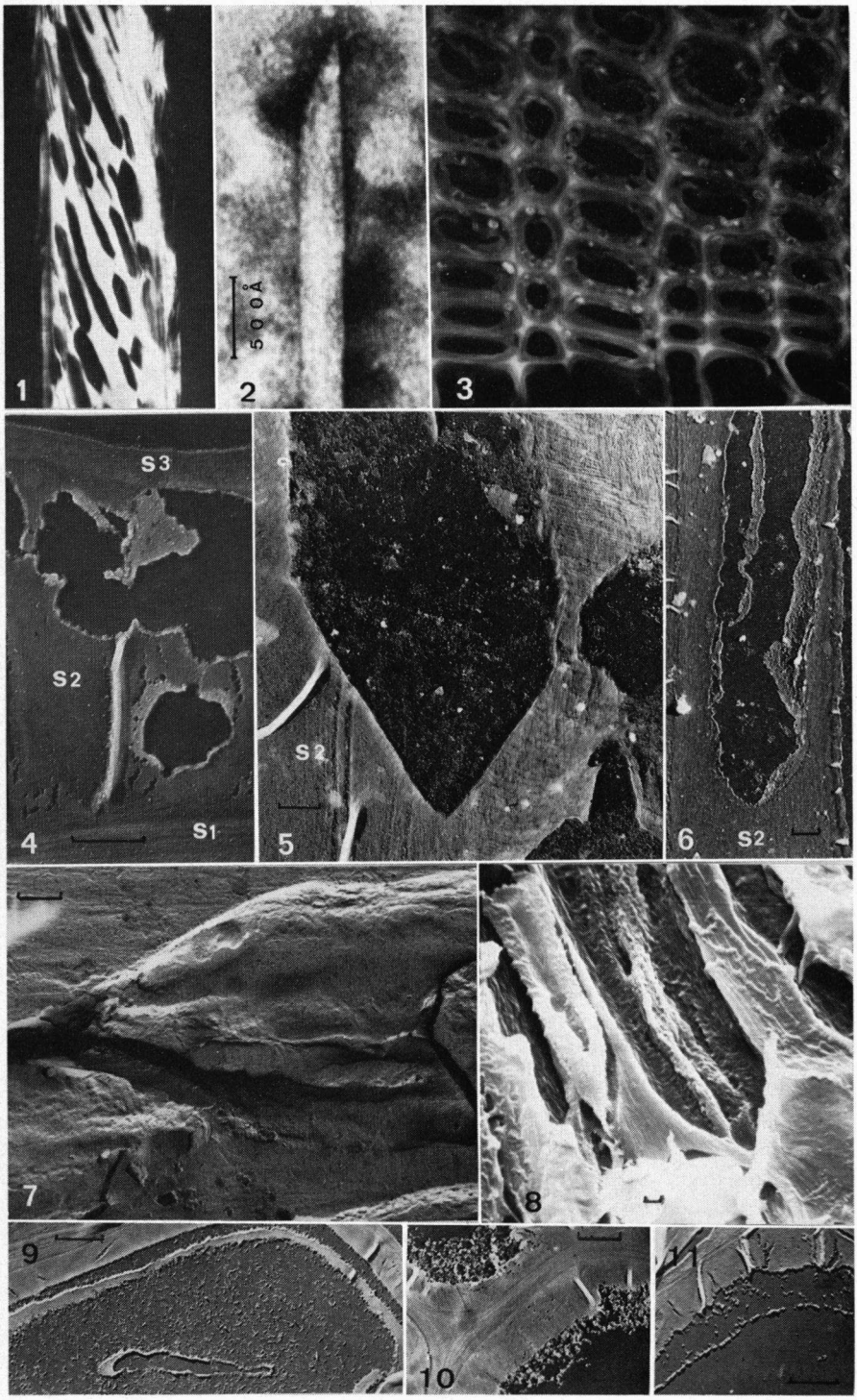
Fig. 7. Replica of longitudinally split wood with the conical end of a cavity and parts of fungi. Electron micrograph.

Fig. 8. Tangential section showing the S2 layer with an open cavity (centre); on its left a cavity which is partly covered by the S3 layer. Scanning electron micrograph.

Fig. 9. *Fagus sylvatica* L. (Beech). Transverse section of tension wood treated with Helicase (43 hrs.). The gelatinous layer is partly digested, showing microfibrils. The layer lining the lumen holds out much longer. Electron micrograph; araldite embedding.

Fig. 10. *Fraxinus excelsior* L. (Ash.). Transverse section of tension wood treated with helicase (2 weeks). The gelatinous layers show disintegration into particles proceeding from the lumen towards the S1 layer. Electron micrograph; methacrylate embedding.

Fig. 11. *Fraxinus excelsior* L. (Ash). Transverse section of tension wood treated with *Trichoderma* cellulase (3 weeks). The gelatinous layer shows degrading into particles, around the lumen a more resistant "pearl rope" is present. Electron micrograph; araldite embedding.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small chips in a mixture of hydrogen peroxide and glacial acetic acid (1:1) for 30–60 minutes in a boiling waterbath, until the original yellow colour had disappeared.

Artificial lignification of isolated cells and parts of the vesicles of *Valonia ventricosa* was carried out using the method of SOLBERG & HIGINBOTHAM (1957). The specimens were immersed in a solution of horseradish peroxidase (Nutritional Biochemicals Corporation, Cleveland, Ohio) for two hours. They were then removed by centrifugation, air dried and washed in distilled water. After washing the specimens were suspended in a mixture of eugenol and hydrogen peroxide in accordance with the procedure of SOLBERG & HIGINBOTHAM (1957) and subsequently subjected to microscopic examination, while immersed in enzyme preparations.

2.4. Examination of specimens by electron microscopy

Small tangential longitudinal sections (60–80 μ thickness) were embedded in either a mixture of butyl and methyl methacrylate (NEWMANN *et al.* 1949) or in araldite (GLAUERT & GLAUERT 1958) before sectioning with an LBK ultratome. The polymerized methacrylate was removed by immersing the sections in chloroform. Araldite was removed from the sections by digestion in sodium methoxide (MAYOR *et al.* 1961). Sections from which the embedding medium was removed were shadowcast with platinum-palladium at an angle of 15–20° to the section surface.

To study the influence of enzymes on the structure, the sections were immersed in enzyme preparations. They were periodically removed and after repeated rinsing in water were embedded in methacrylate or araldite as described before.

Replicas were prepared by a single stage process as mentioned in WARDROP & HARADA (1963). Specimens were examined using an Elmiskop IA electron microscope.

For scanning electron microscopy, sections 15–20 μ in thickness were cut on a sliding microtome from which small pieces were mounted on the specimen holders for the instrument and shadowcast with gold prior to examination in a Stereoscan instrument at the Defence Research Laboratories, Maribyrnong, Melbourne. The results were recorded with a Polaroid Land camera.

3. RESULTS AND DISCUSSION

3.1. The morphology of natural erosion patterns caused by a "soft rot" fungus

The cavities developed in tracheids of Scots Pine infected with an unidentified soft rot fungus showed the same general features as described by BAILEY & VESTAL (1937) and COURTOIS (1963). When macerated and examined between crossed nicols, the cavities in the walls had the same geometrical form and the angle of intersection of the two principal planes was found to be between 23° and 25°. In the very first stages of attack the cavities were approximately circular in transverse section, as long as only a single hypha was involved in the attack.

When attack was in progress the small holes coalesce, leaving large cavities in the layer S2. It was confirmed, as was found by other authors (CORBETT 1963; LEVY 1965) that the layers S3 and S1 were attacked much later. The middle lamella and primary wall, however, did not show any attack. In addition, in fluorescence photomicrographs such as *fig. 3*, the hyphae of the fungus could be seen and the level of fluorescence of the cell wall appeared less than in adjacent uninfected areas, suggesting that the fungus attacked not only the cellulose but also the lignin.

According to LEVI (1965) who studied Beech attacked by soft rot, it can be postulated that the rate of decay is governed by lignin modification, rather than by cellulose decomposition. He found that in attack only a relatively small proportion of the lignin was removed, but that the remaining lignin was definitely modified. Thus, methoxyl groups were preferentially eliminated from the lignin and the residual lignin became increasingly soluble in dilute alkali. He concluded that it seems possible that demethylation may be an essential step in the breakdown of wood. If it is a question of demethylation of lignin, then lignin with a lesser amount of methoxyl groups probably should be attacked more heavily. It may thus be of interest to investigate how compression wood reacts to the attack of soft rot fungi. It is known that the lignin content of compression wood is higher than that of normal wood (TIMELL 1965), but the lignin in compression wood differs in that its methoxyl content is less than that occurring in normal softwood. According to BLAND (1961) even methoxyl free nuclei were found in compression wood.

In radial longitudinal section the cavities found did not appear to have tapered ends, although in the photomicrographs obtained evidence for this was not conclusive. This might have resulted from the lamellated structure of the wall layers in the radial plane. Thus, in investigation of soft rot cavities in the light microscope difficulties arose which prevent the establishment of a clear picture of the exact form of the cavities. One of the difficulties mentioned by BAILEY & VESTAL (1937) is that there is considerable uncertaity in determining whether a particular cavity is being viewed in a truly median longitudinal plane of section.

As could be anticipated from sections seen in the optical microscope in electron micrographs the cavities at first appeared nearly circular in transvere section (*fig. 4*). However, in the region surrounding the cavities the wall components appeared to be less densely packed than in other regions of the wall. It would appear reasonable to interpret these regions to be ones in which degradation was in progress either through the breakdown of lignin or matrix components, or through the removal of cellulose microfibrils. In longitudinal sections the angular form of the cavities could be seen. As there is only a slight chance to cut a truly median section through the conical region of the cavities (*fig. 5*), most of the electron micrographs obtained did not show these features (*fig. 6*). It was of interest to note that the cavities, when examined in the electron microscope, contained a considerable amount of material which was assumed to be degraded lignin and matrix components.

Where sections were cut through the conical region of the cavities, the extreme regularity of the erosion of the wall lamellae was apparent, as was observed in earlier electron micrographs of MEIER (1955). The microfibrils could not be resolved because of the presence of matrix components and lignin. As decay proceeded the cavities became larger and coalesced until the whole S2 layer disappeared. This was also observed by CORBETT & LEVY (1963) and LEVI (1965).

Using the replica method, only in very few cases could the outline of the cylindrical cavities with the cones on both ends be observed. The cavities appeared to have friable edges (*fig.7*).

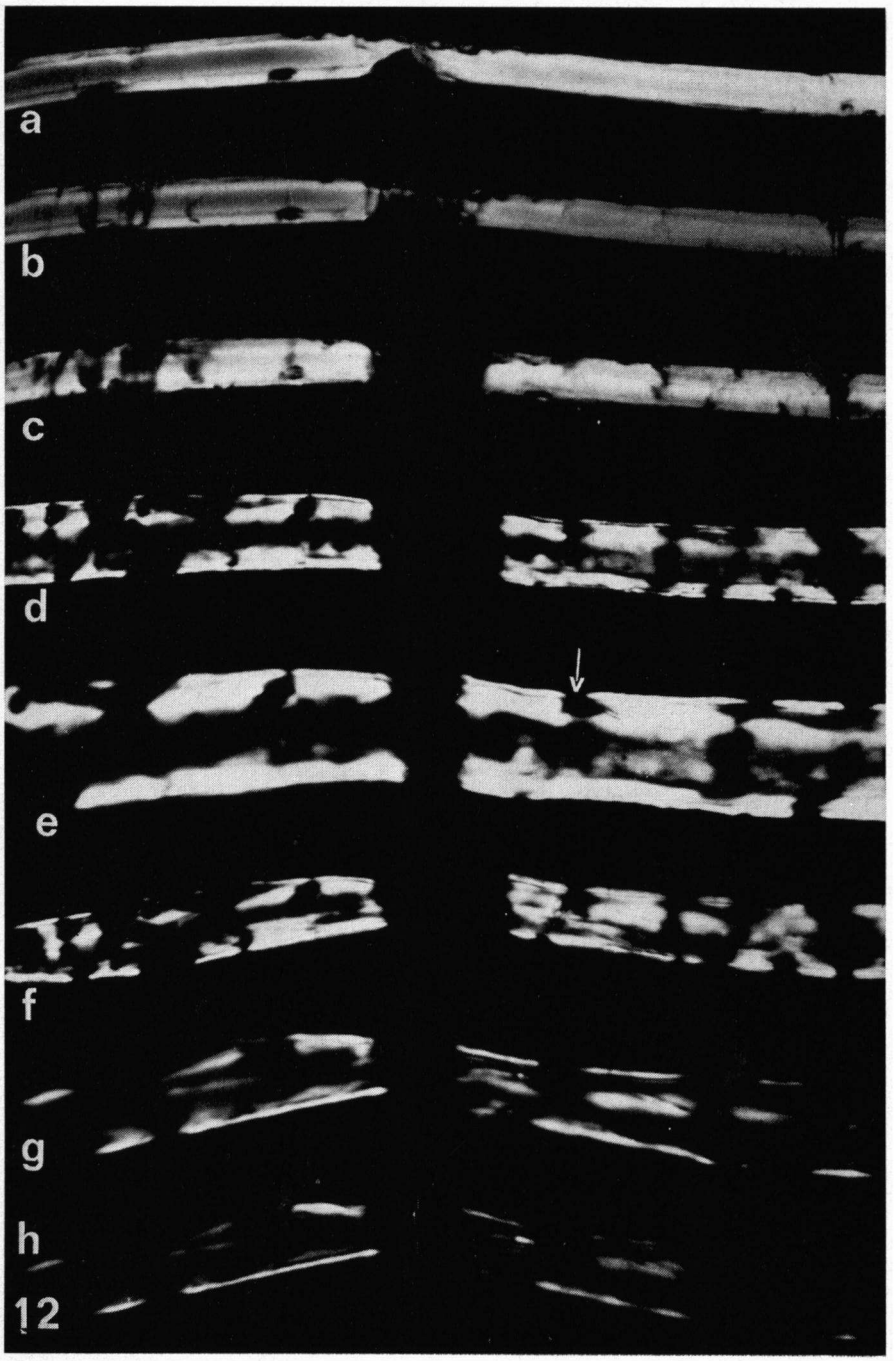
Scanning electron micrographs of sections showed the form of the erosion cavities in the S2 layer, which sometimes could be seen through the overlying layer S3. In contrast to their appearance in the thin sections used in transmission electron micrographs, the edges of the cavities appeared ragged and irregular. This appearance can be interpreted in terms of the thickness of the section, since the lamellae of the cell wall nearest to the surface of the specimen would partially overlie those deeper in the erosion cavity. Furthermore, the method of preparation used for the scanning microscope involves drying the specimen which could be expected to result in some distortion (*fig. 8*).

3.2. The effect of cellulase preparations on xylem and isolated tracheids and fibres of both normal wood and tension wood

In transverse sections studied in the electron microscope the normal xylem fibres of both Beech and Ash wood were not attacked by the cellulase preparations. Even after three weeks no attack could be observed on any of the cell wall layers. The transverse sections of the tension wood, however, showed a considerable erosion even after a short immersion in either of the enzyme preparations. In Beech the erosion pattern is different from that of Ash. In Beech the layer around the lumen was always the most resistant and withstood attack much longer than the thick gelatinous layer, which is wholly degraded within 3 days to 2 weeks. (*fig. 9*). The erosion starts with the breaking down of the cellulose microfibrils into short needles (*fig. 9*). In Ash the breakdown was not into needles, but intosmall clusters which appeared to be amorphous (*fig. 10*).

In previous studies (MEIER 1955; ZENKER 1962) it was observed that attack by soft rot fungi on Birch wood and on the tension wood of Poplar resulted in the removal of the layer S2 and that a resistant layer surrounding the lumen remained. With Ash tension wood, however, no such layer was observed when it had been immersed in helicase preparations (*fig. 10*), though with the *Trichoderma* preparations often a very thin "pearl rope" layer was the last to disappear (*fig. 11*). Thus the different reactions of tension wood would appear

Fig. 12. *Pinus sylvestris* L. (Scots Pine). Degradation of a macerated tracheid immersed in *Trichoderma* cellulase for up to 5 hours at 38°C. The main attack starts in the window pit (centre), followed by degrading along bordered pits, S1 and S2 layer, respectively. Polarized light, between crossed nicols; in the centre of e, g, h, the distance between fibre parts is somewhat reduced; a, b, c, d, f: 300×, e, g, h: 600×.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to be related to the presence of an S3 layer in the normal wood, and of the denatured remains of the cytoplasm (WARDOP & HARADA 1963). An example of the attack of cellulase on isolated fibres or tracheids is illustrated in *figs. 12 a-h* on Scots Pine.

Initial attack took place in the regions of the pits, at dislocations caused by mechanical damage to the cells. The initiation of attack in the pits most probably reflects the fact that at these points the cell wall is locally thinner and in the pit membranes the microfibrils are less densely packed than elsewhere in the cell wall, so that erosion of the wall would first becomes apparent in these regions. Dislocations can occur naturally in the walls of fibres, as it is here the case with tension wood, but they can be readily induced in fibres and tracheids by bending of the cells during maceration. From the early studies of VON HOHNEL (1884) and those of WARDROP & DADSWELL (1947) dislocations are known to be regions more readily penetrated by stains such as chlor-zinc-iodine or by congo red. Because of the locally increased microfibrillar surface there are also regions of preferential hydrolysis by acids or by acetylation (WARDROP & DADSWELL 1947). In view of these observations it is not surprising that initial attack by cellulase-enzymes on the fibres and tracheids should proceed as mentioned above, at the pits or at dislocations.

Erosion of the fibrils appeared to be related to the helical organization of the secondary cell wall, as is the case in specimens attacked by soft rot fungi (BAILEY & VESTAL 1937). Thus, in *fig. 12* the unreacted (birefringent) sections of the wall can be seen to be aligned in a flat helix, presumably corresponding to the microfibrillar orientation in the S1 layer, whereas at more advanced stages of attack the residual regions of the wall were aligned more nearly parallel to the cell axis and to the direction of microfibrillar orientation in the layer S2. It can be seen that in *figs. 12 d-h* the residual birefringent region had tapered ends. In *fig. 12e* the angle of intersection of the terminal planes was 24°. It will be appreciated that the pattern of erosion of the fibres and tracheids by cellulase, shown in *figs. 12 d-h*, is essentially complementary to that resulting from attack by soft rot fungi. It will also be noted that unreacted regions with tapering ends, such as shown in *figs. 12 f-h*, closely resemble the form of isolated fragments obtained from partially acetylated fibres by HESS (1928).

As can be seen from *fig. 12*, because cellulase attack is initiated at points along the cell length, the cell is fragmented into segments. From this type of observation it might be concluded that degradation proceeds along the direction of the microfibrils in each wall layer. On the other hand, the progressive change in the orientation of the unreacted regions of the wall from a direction corresponding to the microfibril orientation of the S1 layer to that of the S2 layer could mean either that attack proceeds progressively through the cell wall to the regions of greatest packing density adjacent to the lumen (LANGE 1954), or that the cellulase permeates the whole wall, but because of its lesser thickness the S1 layer is first removed. These observations thus do not permit any conclusion as to whether there is lateral attack on the cellulose microfibrils as well as longitudinal attack. In an attempt to resolve this point the path difference of fibres

or tracheids was measured during cellulase attack in regions of the wall which appeared structurally uniform, i.e. neither pits nor dislocations were present. It was found that the optical path difference decreased rapidly before any cavities or visible erosion pattern developed. This result suggests that the microfibrils may undergo both lateral and longitudinal degradation by the cellulase.

3.3. The influence of artificial "lignification" of *Valonia* cellulose and of isolated fibres and tracheids on the pattern of cellulase degradation

The fact that erosion cavities by soft rot fungi are usually confined to the S2 layer of the secondary wall and that cellulase (see above) attacks the gelatinous layers of tension wood more readily than the S2 layer of normal wood suggests that the attack of wood by cellulase is inhibited by lignin in the cell wall and that soft rot fungi must possess the means to remove or modify the relation of lignin to the cellulose microfibrils. This is further supported by the chemical data of COWLING (1965) and of LEVI & PRESTON (1965).

To investigate this point further, cellulose from vesicles of *Valonia ventricosa*, isolated fibres of Beech and Ash, and tracheids of Scots Pine, were artificially "lignified" by the method of SOLBERG & HIGINBOTHAM (1957) described above before being subjected to the action of cellulase. After a period of incubation of 24 hours any residual cellulose and the supernatant solution were examined to detect evidence of degradation. In the samples studied which had been "lignified" no evidence of cellulose degradation was apparent. In contrast, the "unlignified" controls showed strong evidence of attack by the cellulase. The fibres and tracheids showed degradation into short fragments similar to that of *fig. 12* and the supernatant showed the presence of microfibrillar aggregates. Cellulose from the vesicle wall of *Valonia ventricosa* became greatly swollen and the supernatant showed microfibrils which frequently had oblique or pointed ends and which showed evidence of breakdown into elementary fibrils as reported in an earlier investigation (WARDROP & JUTTE 1968). It is of interest that the lignified *Valonia* cellulose showed no evidence of attack, even after six months in these enzyme preparations. It was noted further that thin sections (3 μ) of normal wood of Beech and of Ash, which were extracted either with 0.1 M sodium hydroxide or 0.1 M hydrochloric acid, remained resistant to attack by cellulase.

4. GENERAL DISCUSSION

From the above observations it may be concluded from the decrease in the level of fluorescence during attack by soft rot fungi, the eroded appearance of the edges of the cavities, and the effect of artificial lignification on the attack by cellulase on isolated cellulose that the physical or chemical relation between cellulose and lignin must be changed during degradation of wood by soft rot fungi. This evidence is in agreement with chemical studies (LEVI & PRESTON 1965). Since weak acid and alkali hydrolysis of wood did not enhance attack by

cellulase, it may be that the level of lignification rather than the nature of the chemical linkage of lignin to cellulose may be the factor determining whether cellulase can attack the cellulose microfibrils of the wall. As can be seen from *figs. 9–12*, cellulase preparations cause degradation of the cell walls of fibres and tracheids and the fragments remaining from the degraded walls resemble in form the cavities produced by soft rot fungi in wood and the attack appears to be related to the microfibril orientation in the different layers of the secondary wall.

However, the attack on the cell walls proceeds more uniformly over the cell surface, beginning at pits and wall dislocations. It may be noted that if in such cells there were desintegrated regions such as the one shown by the arrow in *fig. 12e* this could well give rise to particles with pointed ends, such as those isolated by HESS (1928) following acetylation of wood fibres. Although the erosion cavities shown in *fig. 12* show only slightly the regularity of the cavities produced by soft rot fungi, their geometrical form suggests that similar factors may operate in their formation. These observations, taken in conjunction with the formation of oblique or pointed ends in individual microfibrils of *Valonia*, suggest that an explanation for the development of the form of soft rot cavities must be sought in the crystalline nature of the cellulose microfibrils.

ACKNOWLEDGEMENTS

This work was carried out while the first author was in receipt of a General Motors Holden's Scholarship. The assistance provided by this scholarship is gratefully acknowledged. The authors wish to thank Mr. F. J. Daniels, Mr. G. M. Harwood and Mr. S. Silva for assistance during the investigation.

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